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Hi,

I would like to request the following alignments:

1. seq id 66 against seq id 2, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 61, 55, 57, 59, and 48.
2. GenBank's AF135484 and AF022462
3. the proteins corresponding to GenBank's AF022462 and AF135484 against seq id 2, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 61, 55, 57, 59, and 48.

Thank you,

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Reaction mechanism of oxidative rearrangement of flavanone in isoflavone biosynthesis

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Microsomes that were prepared from elicitor-treated *Pueraria lobata* cell cultures catalyzed the conversion of liquiritigenin, a flavanone, into daidzein, an isoflavone. The reaction was resolved into two steps. 2, 7, 4'-Trihydroxyisoflavanone was formed as a major product when liquiritigenin was incubated with carefully washed microsomes in the presence of NADPH. The structure of 2, 7, 4'-trihydroxyisoflavanone was confirmed by mass and ^1H NMR spectroscopies. The enzyme responsible for this rearrangement reaction is a cytochrome P-450-dependent monooxygenase. Upon treatment with a soluble enzyme fraction 2, 7, 4'-trihydroxyisoflavanone yielded daidzein quantitatively. The incorporation of ^{18}O from $^{18}\text{O}_2$ into the 2-hydroxy group of 2, 7, 4'-trihydroxyisoflavanone was demonstrated by the shift of molecular ion in its mass spectrum. Based on these observations a new reaction mechanism, hydroxylation associated with 1,2-migration, is proposed for the oxidative rearrangement reaction catalyzed by the cytochrome P-450 enzyme of *Pueraria lobata*.

Pueraria lobata; Isoflavone synthesis; Isoflavonoid; Cytochrome P-450; Rearrangement; Elicitor

1. INTRODUCTION

Isoflavonoids are phenolics widely distributed among higher plants and exhibit a wide variety of biological activities [1]. In particular, leguminous plants produce isoflavonoid phytoalexins as defense substances against phytopathogenic microorganisms [2]. An enzyme that catalyzes the rearrangement reaction of flavanone is the first enzyme of isoflavonoid biosynthesis which branches from the general flavonoid pathway [3-5]. Several hypotheses were proposed for the reaction mechanism of this oxidative 1,2-aryl migration reaction [6]. Kochs and Grisebach reported the isolation of 2-hydroxyisoflavanone as an intermediate in isoflavone synthesis by microsomes from elicitor challenged soybean cells, and proposed a mechanism which involves an enol epoxide of flavanone as a reaction intermediate [3,4]. Crombie et al. suggested another mechanism in which epoxidation occurred on the side phenyl group of flavanone [7]. To clarify the reaction mechanism a microsomal preparation obtained from the elicitor challenged cell cultures of *Pueraria lobata* was studied in detail. This paper deals with the spectroscopic investigations of 2-hydroxyisoflavanone and discusses the results of incorporation experiments with $^{18}\text{O}_2$ gas and ^{18}O -labeled liquiritigenin. A new reaction mechanism, hydroxylation associated with 1,2-aryl migration, will be proposed for the P-450-mediated reaction of isoflavone synthesis.

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2. MATERIALS AND METHODS

2.1. Materials and spectroscopies

$^{18}\text{O}_2$ gas was obtained from ISOTECH Inc. [^{18}O]liquiritigenin was prepared by an exchanging method [8]. The ratio of ^{18}O and ^{16}O at C-4 was determined by mass spectroscopy as 45:55. Mass of NMR spectra were taken on a Hitachi M-80 mass spectrometer and on a JEOL GX-500 apparatus, respectively.

2.2. Cell cultures, elicitor treatment and enzyme preparation

Cell suspension cultures of *Pueraria lobata* were established and challenged with the elicitor as described in a previous communication [5]. The elicitor-challenged cells (100 g fresh weight) were ground in a mortar together with sand (50 g) and polyvinylpyrrolidone (10 g) and buffer (150 ml, pH 7.5) consisting of potassium phosphate (0.1 M), 2-mercaptoethanol (14 mM) and sucrose (10%). The mixture was filtered through nylon gauze and the filtrate was stirred for 20 min with Dowex 1 \times 2 (20 g) pre-equilibrated with the same buffer. After centrifugation at $20\,000 \times g$ for 30 min, the supernatant was further ultracentrifuged at $105\,000 \times g$ for 60 min. The supernatant obtained was used as the soluble enzyme fraction. The pellet was taken up in 20 ml of the same buffer and the slurry was homogenized in a glass homogenizer to give the microsomal fraction. To wash out the contaminated soluble enzymes, the microsomal fraction obtained was re-ultracentrifuged for 60 min at $105\,000 \times g$. Pellet was resuspended in the same buffer at a final concentration of 0.2-0.6 mg protein/ml and used as the washed microsomal fraction.

2.3. Enzyme assay

Incubation experiments with washed and unwashed microsomes were carried out as described in a previous paper [5]. The products formed were analyzed by reverse-phase HPLC (TSK gel ODS-120A, 4.6×250 mm, Tosoh) with a solvent system of acetonitrile/water/acetic acid (19:57:4) at a flow rate of 0.8 ml/min. Retention times (min) of 2,7,4'-trihydroxyisoflavanone, liquiritigenin and daidzein were 8.9, 23.0 and 24.5, respectively.

2.4. Isolation of 2,7,4'-trihydroxyisoflavanone

7.0 μmol of DL-liquiritigenin was incubated for 20 min at 30°C

with 35 μ l of the washed microsomal fraction containing 35.0 μ mol of NADPH. The reaction products were extracted with ethyl acetate and applied to a cellulose plate (Funakoshi), which was developed with 15% acetic acid. The corresponding zone of 2,7,4'-trihydroxyisoflavanone, $R_f = 0.57$, was scraped off and eluted with ethyl acetate. It was further purified with HPLC performed with the same condition as described above.

2.5. Incorporation experiments with $^{18}\text{O}_2$ gas and [4- ^{18}O]liquiritigenin
3.2 μ mol of DL-liquiritigenin and 16.0 μ mol of NADPH were added to 16 ml of washed microsomal fraction in a 50 ml flask which was closed tightly with a rubber septum. The mixture was frozen in a dry-ice/acetone bath and air in the flask was removed by a vacuum pump through an injected needle. The mixture was thawed in a water bath at 4°C. The degassing procedure by freeze and thawing was repeated several times. $^{18}\text{O}_2$ gas was introduced through a needle to the reaction mixture (in a control experiment air was introduced), which was incubated for 20 min at 30°C. The reaction products were extracted with ethyl acetate and subjected to HPLC separation. Labeled 2,7,4'-trihydroxyisoflavanone was analyzed by mass spectroscopy.

In the incorporation experiments of $^{18}\text{O}_2$ gas and [4- ^{18}O]liquiritigenin into daidzein, unwashed microsomes were used. The daidzein formed was purified with HPLC and submitted for mass spectral measurement.

3. RESULTS

3.1. Characterization of 2,7,4'-trihydroxyisoflavanone

When liquiritigenin, 7,4'-dihydroxyflavanone, was incubated with a microsomal fraction prepared from the elicitor challenged cell cultures of *P. lobata* in the presence of NADPH, the only product formed was daidzein, 7,4'-dihydroxyisoflavone [5]. However, another compound was detected as a major product in the reaction mixture which was incubated with a carefully washed microsomal fraction. This compound is an intermediate of the reaction, since it was almost quantitatively converted into daidzein by the soluble enzyme fraction (data not shown). A sufficient amount of the intermediate required for NMR spectroscopy was prepared by a large scale incubation. The EI mass spectrum of the intermediate gave a M^+ peak and an intense $[\text{M}-\text{H}_2\text{O}]^+$ peak at m/z 272 (10%) and 254 (54%), respectively. From the chemical and mass spectral data the compound was identified as 2,7,4'-trihydroxyisoflavanone. ^1H NMR spectrum of 2,7,4'-trihydroxyisoflavanone showed two series of signals arising from two stereoisomers (I and II, see Table I) in a ratio of 3:1 in d_6 -acetone solution. The complete assignments of NMR signals based on decoupling experiments are shown in Table I.

3.2. Participation of cytochrome P-450

The conversion of liquiritigenin into 2,7,4'-trihydroxyisoflavanone by the washed microsomal fraction required NADPH and atmospheric oxygen. The reaction was inhibited by typical P-450 inhibitors as well as carbon monoxide, indicating that the enzyme responsible for this reaction is a P450 (Table II). Dehydratase that converted 2,7,4'-trihydroxyisoflavanone into daidzein was not affected by P-450 inhibitors (data not shown).

Table I

^1H NMR data for 2,7,4'-trihydroxyisoflavanone (d_6 -acetone, 500 MHz)

Proton no.*	Isomer (I)		Isomer (II)	
	ppm	J (Hz)	ppm	J (Hz)
2	5.91d	3.1	5.86d**	3.1
3	3.77d	3.1	4.22d	3.1
5	7.52d	8.6	7.76d	8.6
6	6.63dd	8.6, 2.1	6.62dd	8.6, 2.1
8	6.46d	2.1	6.46d	2.1
2',6' [2H]	7.18d	8.6	7.26d	8.6
3',5' [2H]	6.81d	8.6	6.83d	8.6

*Numberings were shown in Fig. 2

**Broad doublet

3.3. The origin of the 2-hydroxy group of 2,7,4'-trihydroxyisoflavanone

An incorporation experiment with $^{18}\text{O}_2$ gas has clearly demonstrated the origin of the C-2 hydroxyl of 2,7,4'-trihydroxyisoflavanone. As shown in Fig. 1, the labeled 2,7,4'-trihydroxyisoflavanone gave a shifted molecular ion peak by 2 mass units at m/z 274, while the dehydrated fragment peak appeared at m/z 254 without any shift. It was concluded from these results that ^{18}O atom of $^{18}\text{O}_2$ gas was incorporated into the 2-hydroxyl of 2,7,4'-trihydroxyisoflavanone in an incorporation ratio of ca 30%.

3.4. Retention of carbonyl oxygen of liquiritigenin during 1,2-aryl migration

The mass spectrum of daidzein obtained from an incubation experiment with [4- ^{18}O]liquiritigenin showed a ratio of $^{16}\text{O}:^{18}\text{O}$ which was exactly the same as in the

Table 2

Effect of inhibitors for cytochrome P-450 enzyme on 2,7,4'-trihydroxyisoflavanone synthesis

Inhibitor	Conc. (μ mol/l)	Inhibition (%)
None	-	0
SKF 525-A	2000	73
	1000	44
	100	8.5
Ancyridol	1000	100
	100	91
	10	65
	1	5
Uniconazole	100	100
	10	89
	1	33
Metyrapone	100	100
	10	85
	1	35
CO gas	-	52

starting material (45:55). This indicates that the carbonyl oxygen of liquiritigenin was not exchanged during the reaction. As expected, the incorporation of ^{18}O from $^{18}\text{O}_2$ gas was not observed into the 4-carbonyl of daidzein.

4. DISCUSSION

As suggested by Kochs and Grisebach [4] the reaction catalyzed by the microsomal preparation of *P. lobata* which converts liquiritigenin to daidzein consists of two reaction steps. In the first step, liquiritigenin is converted into 2,7,4'-trihydroxyisoflavanone by a microsomal P-450-dependent monooxygenase. In the second step, 2,7,4'-trihydroxyisoflavanone is dehydrated to yield daidzein by a dehydratase which is a soluble enzyme contained in unwashed microsomal preparations. The $^1\text{H-NMR}$ spectrum indicates that 2,7,4'-trihydroxyisoflavanone exists as a mixture of two stereoisomers in d_6 -acetone solution. A facile epimerization at C-3 would generate isomers at C-3, while the isomerization of C-2 configuration would be also possible through a hemiacetal and an aldehyde equilibrium. Due to the lack of data the exact stereochemistries of the isomers are unknown.

It was unambiguously demonstrated that ^{18}O of molecular oxygen was incorporated into the 2-hydroxy group of 2,7,4'-trihydroxyisoflavanone. A relatively low incorporation ratio (ca 30%) may be attributable to an exchanging reaction through free aldehyde, and also to an exchange of oxygen bound to P-450 porphyrin iron with water [9]. If the enol epoxide of flavanone is involved in the rearrangement reaction, a ketal hydroxyl that was derived from the epoxide oxygen had to migrate stereospecifically from C-4 to C-2 to give the

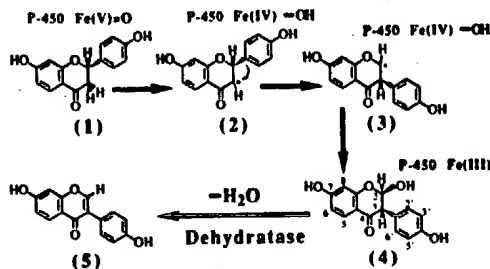


Fig. 2. Proposed reaction sequence from liquiritigenin (1) to daidzein (5) via 2,7,4'-trihydroxyisoflavanone (4).

hydroxyl of 2,7,4'-trihydroxyisoflavanone. This is highly unlikely, since migration should occur via a four-member ring ether with a hemiketal and acetal structure. A new reaction mechanism of cytochrome P-450 hydroxylation has emerged from these observations (Fig. 2). The hydroxylation reaction of cytochrome P-450 normally proceeds by the abstraction of a hydrogen followed by bond formation with hydroxy radical at the same carbon atom [10]. In the case of 2,7,4'-trihydroxyisoflavanone, the abstraction of a hydrogen at C-3 of liquiritigenin is followed by the 1,2-shift of phenyl group which results in forming a carbon radical at C-2. Hydroxylation takes place at C-2 to give 2,7,4'-trihydroxyisoflavanone. The bond formation of the hydroxy radical to the migrated radical center has been observed in the oxidation reactions of cyclohexene, methylenecyclohexane, and β -pinene with a P-450 [11]. The hydroxylation associated with rearrangement is a new category in P-450 reactions, though an underlying reaction, hydrogen abstraction and the ensuing hydroxylation, is typical for P-450. Many biosynthetic reactions are accounted for by this mechanism. For example, the ring contraction of 7-hydroxykaurenoic acid to form a gibberellin (GA₁₂ aldehyde) was reported to be catalyzed by a microsomal preparation and NADPH [12]. The reaction was explained by 1,2-migration followed by hydroxylation, exactly the same mechanism as mentioned above, though there is no evidence that the reaction is catalyzed by a P-450. Several biosynthetic reactions of this type are under investigation in our laboratory.

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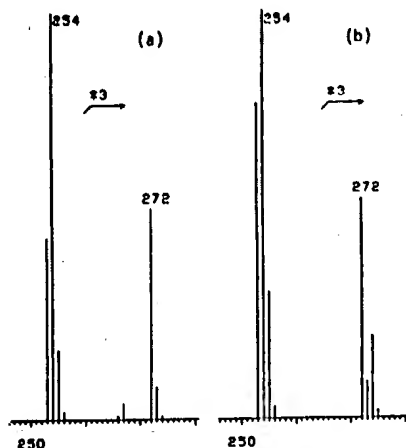


Fig. 1. EI-MS spectral data of enzymatically formed 2,7,4'-trihydroxyisoflavanone in (a) control experiment and (b) $^{18}\text{O}_2$ exchanged system. The peaks larger than m/z 265 were displayed as three-times magnified values.

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